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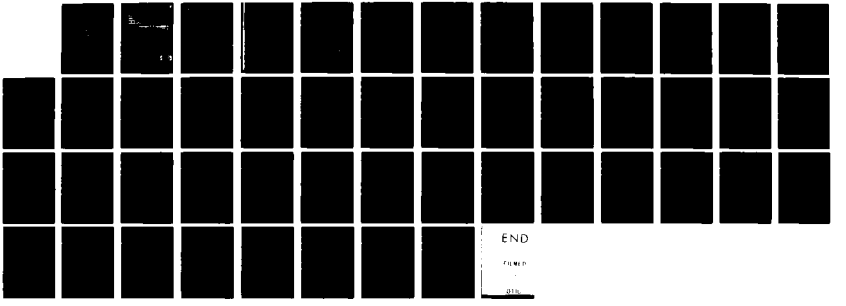

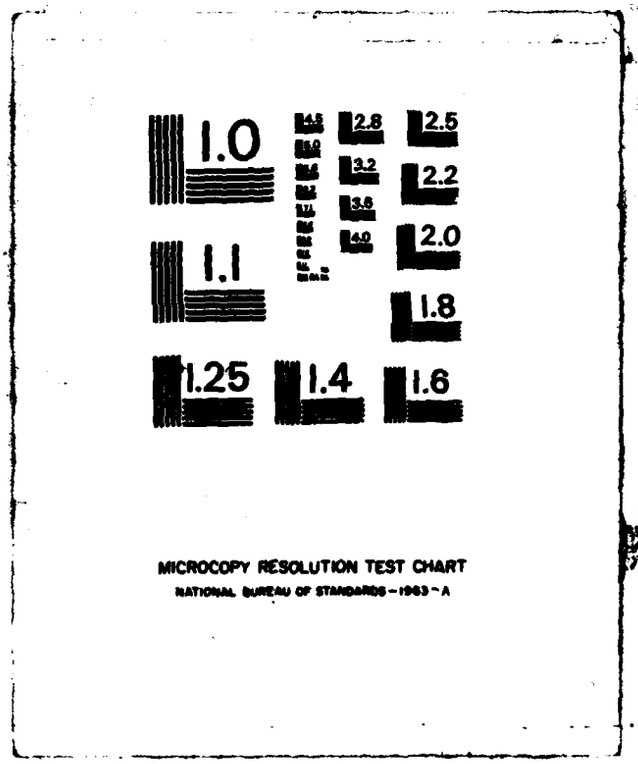
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IMMUNE DYSFUNCTIONS AND ABROGATION OF THE INFLAMMATORY RESPONSE BY ENVIRONMENTAL CHEMICALS

Richard G. Olsen
Department of Veterinary Pathobiology

For the Period
July 1, 1981 - June 30, 1982

AIR FORCE OFFICE OF SCIENTIFIC RESEARCH
Bolling Air Force Base, D.C. 20332

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1,1-dimethylhydrazine (UDMH); suppressor cells; macrophages; mixed lymphocyte reaction; prostaglandins

20. ABSTRACT (Continue on reverse side if necessary and identify by block number)

During the past year we have further characterized the immunotoxic effects of 1,1-dimethylhydrazine (UDMH). Evidence has been accumulated which indicates that UDMH interferes with the regulatory arm of the immune system at low dose or concentration levels.

Results from earlier experiments which supported this conclusion included: increased Jerne plaque response in UDMH-treated mice; abrogation of concanavalin

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Block 20 (Abstract) - Continued

A-induced suppressor activity following in vivo or in vitro UDMH exposure; and enhancement of lymphocyte blast transformation (LBT) by UDMH-treated lymphocytes. During the past year we have shown that at least one type of regulatory cell which UDMH appears to inhibit is in the adherent cell population of splenocytes. This is demonstrated by the fact that exposure of adherent cells to UDMH enhanced the mixed lymphocyte reaction (MLR). Preliminary experiments now in progress indicate that the UDMH suppresses prostaglandin E (PGE) production by the adherent splenocyte population, which would explain the effects seen in the MLR, as PGE has been shown by others to inhibit various immune responses, including the MLR. Experiments are currently underway which will evaluate the effects of UDMH on PGE synthesis by various subpopulations of lymphocytes and macrophages, as well as on cyclic nucleotide levels and Ia antigen expression (Ia antigen is a product of the immune responder genes).

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For the period July 1, 1981 - June 30, 1982

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Progress report

I. Research objectives

During the FY 1981, 1982, we continued to characterize the immunotoxic effects of 1,1-dimethylhydrazine (UDMH), and have begun attempts to delineate the mechanisms of its effects at a cellular and molecular level. We are focusing on the mechanism of action of UDMH in the hope that it will eventually lead to some "common denominators" which will explain seemingly diverse immunotoxic effects, and even toxic effects on other organ systems.

Specific objectives for FY 1981-82 included:

A. Completion of the evaluation of effects of UDMH on adherent and non-adherent responder cell populations and intact stimulator cell populations in the mixed lymphocyte reaction (MLR).

B. Completion of evaluation of UDMH on macrophage function (phagocytosis, killing, chemotaxis) using thioglycollate-elicited peritoneal macrophages.

C. Finish experiments on effects of UDMH on development of autoimmune disease in MLR mice.

D. Begin evaluation of UDMH on expression of lymphocyte membrane markers (Lyt 1.2, Lyt 2.2).

E. Begin evaluation of UDMH on membrane expression of Ia antigens on lymphocytes and macrophages.

F. Begin evaluation of UDMH on prostaglandin synthesis by macrophages and lymphocytes.

G. Begin evaluation of UDMH on cyclic nucleotide levels in macrophages and lymphocytes.

II. Status of Research

A. In vitro UDMH treatment of cell subpopulations in the murine mixed lymphocyte reaction (MLR).

The in vito and in vitro effects of UDMH on the murine MLR were studied. In vivo studies in which splenocytes from mice injected with UDMH for one week were used as the responder cell population showed a significantly reduced MLR with doses of 10 µg UDMH/kg mouse ($p \leq .01$), 25 µg/kg ($p \leq .025$) and 50 µg/kg ($p \leq .005$). The in vitro addition of UDMH throughout the MLR 7 day culture period resulted in a concentration related suppression of the MLR response at concentrations of 10 µg/ml ($p \leq .01$), 25 µg/ml ($p \leq .005$), and 50 µg/ml ($p \leq .025$). A similar suppression was noted in assays in which adherent cells had been removed from the responder cell population and UDMH added for the duration of the culture period. Decreases in responses were observed at 25 µg/ml ($p \leq .025$) and 50 µg/ml ($\leq .050$). Two hour pre-treatment with UDMH on various cell sub-populations involved in the MLR produced the following results: 1) pre-treatment of nonadherent responder cells (without reconstitution with adherent cells) resulted in a slight concentration related suppression of MLR at concentrations of 10 µg/ml ($p \leq .025$), 25 µg/ml ($p \leq .10$) and 50 µg/ml ($p \leq .05$); 2) pre-treatment of adherent cells in the responder cell population resulted in a slight enhancement of MLR 50 µg/ml($p \leq .05$), and a more pronounced enhancement when compared to treated intact responder cells; 3) pre-treatment of intact responder cell population, treated non-adherent cells reconstituted with untreated adherent cells, and intact stimulator cells did not alter MLR. There was no cytotoxicity due to UDMH treatments observed in any assays. These data indicate that UDMH in some manner modifies specific immune cell functions. (See enclosed reprint for methodology details and tables of results).

B. Effects of in vitro UDMH treatment on macrophage function.

Experiments have been done to evaluate the effects of UDMH on the phagocytic, microbicidal, and chemotactic properties of macrophages. The source of macrophages was peritoneal exudate cells (PEC), which were elicited by injecting Balb/C mice with thioglycollate intraperitoneally, then harvesting the PEC four days later. To determine phagocytic and microbicidal functions, PEC ($1x10^6$) were added to live yeast cells (cell to yeast ratio of 1:5) and incubated for 2½ hours at 37°C in the presence of methylene blue dye and varying concentrations of UDMH. The cell-yeast mixture was then pipetted onto a hemocytometer chamber and the percent of PEC containing at least two live (unstained) or dead yeast (percentage phagocytosis), as well as the percent of PEC containing at least one killed (denoted by blue dye) yeast (percentage killing), were determined. The results as shown in Tables 1 and 2 indicate that UDMH significantly decreases the amount of phagocytosis of yeast by the PEC at concentrations of 5 to 100 µg/ml UDMH, however there is no alteration in phagocytosis seen at concentrations of UDMH at 200 µg/ml. At concentrations of 100, 200, and 300 µg/ml UDMH there is noted a decrease in microbicidal activity of the PEC.

The chemotactic activity of the PEC was determined by the use of a 48 well micro chemotaxis chamber. PEC ($2x10^5$) and varying concentrations of UDMH were placed in the upper wells of the chamber. The chemoattractant used was zymosan activated by complement (present in fresh mouse sera). The zymosan was placed in the lower wells of the chamber and separated from the upper wells by a 5 µ pore size filter. The chamber was incubated for 5 hrs. at 37°C and the PEC allowed to migrate into the filter paper barrier between the

upper and lower chambers. After this 5 hr. period, the filter paper was stained with Hemal-Stain and examined microscopically. Results are expressed as the number of cells which migrated through the filter per ten high power fields (Table 3). The presence of UDMH in this assay system neither significantly inhibited nor enhanced the chemotactic activity of the PEC, however a slight to moderate decrease was noted at all concentrations of UDMH tested.

C. Effects of UDMH treatment on the development of autoimmune disease in MRL-lpr/lpr mice

Previous experiments indicated that UDMH treatment (25 mg/kg 3 times/-week from 8 weeks of age until death) did not affect age of onset of lymphadenopathy or death in MRL-lpr/lpr mice (a strain of mice which develop autoimmune disease early in life) (Table 4). Subsequent experiments indicated that development of antinuclear antibody was not affected by similar UDMH treatment (Table 5).

D. Effects of UDMH on expression of membrane markers of lymphocyte subpopulations.

These experiments are still in the beginning phase. Initially, splenocytes were incubated with anti-mouse immunoglobulin serum, then passed through a column of protein A-sepharose GMB beads, in an attempt to remove B-cells from the population. The unbound cells (mostly T-cells) passed through the column, and then were incubated with monoclonal anti Ly 1.2 (helper T cells) or anti Ly 2.2 (suppressor/cytotoxic T-cells), followed by FITC conjugated anti-mouse immunoglobulin. Problems were encountered in that too many cells were lost in the column, and the cell populations were not purified adequately. Similar experiments are now being attempted using a direct

immunofluorescent assay, i.e. FITC conjugated monoclonal anti Ly 1 and anti Ly 2 reagents. These reagents will be used on splenocyte suspensions which have been exposed to UDMH, as well as on frozen tissue sections of lymph nodes, spleen and thymus from animals which have been treated with UDMH.

E. Effects of UDMH on Ia antigen expression

The visualization of Ia antigen on adherent and non-adherent cell surfaces has been achieved using an indirect immunofluorescent technique. Live cells are incubated with monoclonal rat anti-mouse Ia reagent for 45 minutes at 4°C. The cells are then washed and treated with rabbit sera for 45 minutes at 4°C. After this incubation period, the cells are again washed and then incubated with rabbit anti-rat immunoglobulin serum for 45 minutes at 4°C. The cells are then washed and counterstained with new methylene blue dye. When viewed with a fluorescent microscope, Ia positive cells have a greenish-yellow membrane fluorescence on their cell surfaces, while Ia-negative cells are counterstained a dull red color. The number of Ia-positive cells can then be quantitated and percentages of Ia-positive cells computed. Experiments are now being initiated in which UDMH will be added to live splenocyte suspensions for variable time periods and then tested for Ia antigen expression, in comparison to Ia antigen expression on non-treated incubated cells.

Also, Ia antigen expression will be evaluated on frozen tissue sections of spleen, thymus, and lymph nodes from UDMH-treated and control mice using the immunofluorescence procedure.

F. Effects of <u>in vitro</u> UDMH on macrophage prostaglandin E_2 and cyclic nucleotide synthesis.

Preliminary experiments have been done to evaluate the effects of UDMH on PGE_2 and cyclic nucleotide (cAMP-cGMP) synthesis and release into culture

media by murine spleen cells. Spleen cells ($5x10^6$/ml) were incubated for 2 and 24 hours at 37° C in the presence of varying concentrations (5-200 µg/ml) of UDMH. Supernatants were collected and then evaluated for PGE_2 and cells were harvested with trichloracetic acid for cAMP-cGMP levels. Levels of these compounds were determined by radioimmunoassay which is set up on the basis of competitive binding, Radioactive labelled PGE_2 and cyclic nucleotides and their antisera counterparts were added to the samples. Labelled PG and cyclic nucleotides compete for binding sites on the antisera with any PG and cAMP-cGMP already present in the samples. Results produced as the means of the percent binding of the labelled PGE_2, cAMP, and cGMP were then plotted on an 11-18 point standard curve for each compound. The standard curve for PGE_2 ranged from 1 picogram (pg) to 10,000 pg/100 µl; for cAMP, from 1.25 to 10,000 fentamoles (fm)/100 µl; and for cGMP, from 1.25 to 10,000 fm/300 µl diluent. Results from the samples tested are expressed for PGE_2 pg/$5x10^5$ cells; for cAMP in fm/$1x10^5$ cells; and for cGMP, in fm/$3x10^5$ cells.

Results from preliminary experiments indicate that UDMH does inhibit PGE_2 synthesis by adherent splenocyte populations. A sample experiment consisted of incubating adherent splenocytes for two hours with UDMH, then harvesting the supernatant and measuring PGE_2 levels. The results shown in Table 6 indicate that the most suppressive effects are seen at concentrations of 25-100 µg/ml UDMH, with less suppression at lower (5-10 µg/ml) and higher (200 µg/ml) concentrations. Other experiments have yielded similar results. PGE_2 synthesis by intact and non-adherent splenocyte populations was not affected by UDMH exposure in preliminary experiments. Further experiments

with different times of exposure, concentration of UDMH, and cell populations are now in progress.

To date, no results are available for experiments involving effects of UDMH on cellular cyclic nucleotide levels.

III Written Publications (cumulative list)

A. Suppression of mitogen-induced blastogenesis of feline lymphocytes by in vitro incubation with carcinogenic nitrosamides. Tarr, M.J. and Olsen, R.G. Immunopharmacology 2:191-199, 1980.

A Differential effects of hydrazine compounds on B- and T-cell immune function. Tarr, M.J. and Olsen, R.G. AGARD Conference Proceedings No. 309, Toxic Hazards in AViation. B3-1-7, 1981.

C. In vivo and in vitro effects of 1,1-dimethylhydrazine on selected immune functions. Tarr, M.J., Olsen, R.G. and Jacobs, D.L. Immunopharmacology 4:139-147, 1982 (reprint enclosed).

D. Species variation in susceptibility to methylnitrosourea-induced immunosuppression. Tarr, M.J. and Olsen, R.G. In press, J. Env. Path. and Toxicol. 1982.

E. Effects of 1,1-dimethylhydrazine on the murine mixed lymphocyte reaction. Jacobs, D.L., Tarr, M.J. and Olsen, R.G. Submitted to Intl. J. Immunopharmacology, 1982.

F. Effects of 1,1-dimethylhydrazine on peritoneal macrophage functions. Tarr, M.J., Jacobs, D.L. and Olsen, R.G. In preparation. To be submitted to J. Immunopharmacology.

IV. Professional Personnel Assocated with Research Effort

Richard G. Olsen, Ph.D., Principle Investigator

Departments of Veterinary Pathobiology, Microbiology (College of Biological Sciences), and Comprehensive Cancer Center, The Ohio State University, Columbus, Ohio 43210

Melinda J. Tarr, D.V.M., Ph.D., Co-investigator

Department of Veterinary Pathobiology, The Ohio State University, Columbus, Ohio 43210.

Debra L. Jacobs, B.S., M.S., Research Assistant

Department of Veterinary Pathobiology, The Ohio State University, Columbus, Ohio 43210. Received M.S. degree from this department in March, 1982. Thesis title: Effects of 1,1-unsymmetrical dimethylhydrazine on the murine mixed lymphocyte reaction. Ms. Jacobs is continuing to work on this contract as a research assistant.

Grace Sutter, B.S. Research Assistant

Department of Veterinary Pathobiology, The Ohio State University, Columbus, Ohio 43210.

V. Oral Presentations

A. Effects of 1,1-unsymmetrical dimethylhydrazine on the murine mixed lymphocyte reaction. Jacobs, D.L., Tarr, M.J. and Olsen, R.G. Second International Conference on Immunopharmacology, Washington, D.C., July, 1982.

B. Comparison of <u>in vitro</u> and <u>in vivo</u> immunotoxicology assays. Tarr, M.J., Olsen, R.G. and Jacobs, D.L. Conference on Cellular Systems for Toxicity Testing, New York Academy of Sciences, New York, N.Y. October, 1982.

Table 1. Effects of UDMH on phagocytosis of peritoneal macrophages.

Concentration UDMH (ug/ml)	Percent phagocytosis[a]	P value[b]
0 (media control)	42.7 ± 4.0	-
5	38.7 ± 3.0	NS[c]
10	32.8 ± 4.0	<.025
25	32.8 ± 4.0	<.025
50	28.2 ± 4.5	<.005
100	35.8 ± 3.5	<.025
200	42.7 ± 4.1	NS
300	37.3 ± 4.0	<.05

[a]Determined by: $\frac{\text{No. macrophages containing 2 or more yeast organisms}}{\text{total no. macrophages}} \times 100$

At least 100 total cells counted per slide. Expressed as percent ± standard error of the mean.

[b]P value determined by student t test.

[c]NS = not significant.

Table 2. Effects of UDMH on microbicidal capacity of peritoneal macrophages.

Concentration UDMH (µg/ml)	Percent macrophages[a] containing dead yeast	P value[b]
0 (media control)	14 ± 2	-
5	16 ± 1.5	NS[c]
10	14 ± 1	NS
25	16 ± 4	NS
50	14 ± 4	NS
100	10 ± 2	<.025
200	10 ± 2	<.05
300	8 ± 1	<.001

[a]Determined by: $\frac{\text{No. macrophages containing 1 or more dead yeast organisms}}{\text{total no. macrophages}} \times 100$

Yeast viability determined by trypan blue dye exclusion.
Expressed as percent ± standard error of the mean.

[b]P value determined by student t test.

[c]NS = not significant

Table 3. Effects of UDMH on the chemotactic properties of peritoneal macrophages.

Concentration UDMH (ug/ml)	No. migrated cells/10 hpf[a]
0 (media control)	338 ± 77
1	310 ± 58
5	275 ± 49
10	242 ± 49
25	251 ± 24
50	270 ± 60
100	307 ± 63
200	252 ± 29

[a]hpf = high power field. Expressed as number of cells which migrated through the filter ± standard of the mean/10 hpf.

Table 4. Effects of 25 mg/kg UDMH on age of onset of lymphadenopathy in MRL-lpr/lpr mice.

UDMH-treated		Control	
Animal No.	Age of onset (days)	Animal No.	Age of onset (days)
females		females	
1	81	1	97
2	97	2	97
3	99	3	105
4	89	4	99
5	102	5	97
mean	93.6	mean	99.0
males		males	
1	110	1	98
2	119	2	119
3	114	3	141
4	145	4	135
5	151		
mean	127.8	mean	123.3

Table 5. Effects of UDMH treatment[a] on development of antinuclear antibody in MRL-lpr/lpr mice.

	Fluorescent Intensity of Sera[b]		
	pre-UDMH treatment	14 dpi[c]	33 dpi
male, 25 mg/kg UDMH	0.8	2.6	3.4
male, PBS control	1.6	3.0	3.5
female, 25 mg/kg UDMH	2.4	3.3	3.5
female, PBS control	2.8	3.6	4.0

[a]Mice were treated with 25 mg/kg UDMH or phosphate buffered saline (diluent control) 3 times/week from 8 weeks of age until death.

[b]Expressed as intensity of fluorescence based on a scale of 0 (no fluorescence) to 4 (maximum fluorescence) using test sera diluted at 1:40.

[c]dpi = days post initiation of UDMH treatment.

Table 6. Effects of UDMH[a] on PGE_2 production by adherent splenocyte population.

Concentration UDMH (µg/ml)	PGE_2 levels in media (pg/100 µl media[b])
0 (media control)	157
5	122
10	148
25	88
50	96
75	91
100	84
200	102

[a]Adherent cells were exposed to UDMH for 2 hours, then the media harvested for PGE_2 assay.

[b]Media was from adherent cells removed from whole splenocyte suspensions plated at $5x10^6$ cells/ml.

VI. Significance of Results to Date and Future Direction.

Work to date has clearly indicated that UDMH modulates the immune response, augmenting the response mainly at low to medium concentration or dose ranges, and inhibiting the response at higher exposure levels. Evidence has been presented that the augmentation of the immune response by UDMH is due to interference of the suppressor or regulator cell mechanisms of the immune system. This observation correlates well with published reports of the association of autoimmune disease with administration of hydrazine-derivative drugs such as hydralazine.

There are several possible mechanisms for this effect, and it has been the elucidation of this (or these) mechanism to which we have directed and will continue to direct our efforts.

Our preliminary experiments involving the effects of UDMH on prostaglandin (PGE) synthesis by adherent cells have shown that UDMH does suppress PGE synthesis, particularly at the concentration ranges that it also inhibits suppressor cell activity. This could be one mechanism of the immunoenhancement properties of UDMH, as there is now good evidence that PGE does suppress the immune response and probably plays an important role in immunoregulation in the in vivo cellular microenvironment. In addition, it has recently been shown that PGE at physiologic concentrations suppresses Ia antigen expression by peritoneal macrophages (D.S. Snyder et al. Nature 299:163-165, 1982). The Ia antigen is a product of the immune responder gene; it is expressed on the membrane of certain subsets of lymphocytes and of macrophages, and its expression and recognition by other subsets is necessary for a normal immune response to occur. Hence, inhibition of its expression would be one way of regulating (suppressing) the immune response.

Conversely, if Ia antigen expression is increased, the immune response would be enhanced. We are now initiating experiments to determine the effects of UDMH on Ia antigen expression by various immunocyte populations.

The importance of our findings to date is two-fold. First of all, regarding exposure to UDMH, it is apparent that chronic exposure to low, subtoxic levels may result in subtle immunoregulatory disorders long after exposure has begun. These effects may not be clinically manifest, or if they are, the signs (e.g. of autoimmune disease) may not be readily linked to the UDMH exposure.

Secondly, by studying the mechanism of action of UDMH, it will be possible to apply the techniques and information learned to immunotoxic evaluation of other compounds. These studies may also contribute to the elucidation of basic mechanisms of the immune system which is now rapidly taking place.

In Vivo and In Vitro Effects of 1,1-Dimethylhydrazine on Selected Immune Functions

Melinda J. Tarr, Richard G. Olsen, and Debra L. Jacobs

Abstract: *The in vivo phase of the experiments reported here include the evaluation of immune function after short- or long-term treatment of mice with 1,1-dimethylhydrazine (UDMH). Long-term exposure (3 injections/week for 14 weeks) resulted in increased numbers of Jerne plaque-forming cells, a trend toward decreased induction of suppressor cell activity by concanavalin A (Con A), and no effects on mitogen-induced lymphocyte blast transformatin (LBT), compared to saline-treated control mice. These effects were greatest at doses of 10 or 50 mg/kg, while higher doses had less of an effect. In vitro experiments were performed by adding UDMH to normal murine splenocytes in the LBT assay and con A-induced suppressor cell assay. The UDMH induced a significant enhanced response to lipopolysaccharide (LPS) at 10 and 50 μg/ml, and a suppressed response to both Con A and LPS at higher concentrations. The UDMH also caused a decrease in suppressor cell activity at 25 μg/ml. Selective abrogation of suppressor activity or alteration of the suppressor cell-helper ratio were suggested as possible mechanisms for the enhancement effect associated with UDMH.*

Key Words: 1,1-Dimethylhydrazine; Lymphocyte blast transformation; Jerne plaque cells; Con A-induced suppressor cells

INTRODUCTION

Numerous environmental pollutants, drugs, or industrial chemicals are now known to suppress or modulate immune functions. Many compounds exert an immunosuppressive effect, often at doses lower than those causing overt toxicity or death. These compounds include polychlorinated biphenyls (Friend and Trainer, 1970; Thomas and Hinsdill, 1978), tetrachlorobenzo-p-dioxin (Faith and Moore, 1977), heavy metals such as lead, cadmium, and mercury (Koller, 1973), and methylnitrosourea (Tarr et al., 1979). Other compounds have been associated with an enhancement of the immune response, or an enhancement at one dose and suppression at higher or more prolonged doses. For instance, mice exposed to NO or NO_2 showed an enhanced antibody response to a T-dependent antigen after 10 weeks of exposure, but a

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52 Vanderbilt Ave., New York, N.Y. Immunopharmacology 4, 139–147 (1982) 0162-3109/82/0213909$02.75

depressed response after 30 weeks (Holt et al., 1979). The same biphasic pattern was seen with graft-versus-host reactions. Exposure of mice to vinyl chloride also resulted in immunostimulation as measured by enhanced spontaneous lymphocyte blastogenesis and enhanced blastogenic response to phytohemagglutinin and pokeweed stimulation (Sharma and Gehring, 1979). Long-term high dose exposures resulted in less enhancement than short-term high dose exposures. These results correlate with the supposition that the vinyl chloride syndrome experienced by exposed industrial workers may involve an autoimmune phenomenon (Ward et al., 1976).

Numerous other drugs have been associated with autoimmune disease, and some of these induce suppressor cell malfunctions, such as methyldopa (Kirtland et al., 1980). Exposures to compounds containing the hydrazine moiety ($-N-NH_2$), such as hydralazine (Perry, 1973) or hydrazine sulfate (Durant and Harris, 1980) have induced systemic lupus erythematosus(SLE)-like signs. The purpose of the experiments reported here was to examine the in vitro and in vivo effects of a simple hydrazine derivative, 1,1-dimethylhydrazine (UDMH), on immune response and suppressor cell induction in mice.

MATERIALS AND METHODS

Animals

Male and female Swiss outbred mice weighing 20–35 g were used for all in vivo or in vitro experiments.

Chemical Preparation

The UDMH was obtained in liquid form (Aldridge Chemical Corp.) and further distilled (Dr. Kenneth Back and associates, Wright Patterson Air Force Base, Dayton, OH). It was diluted in phosphate buffered saline (PBS) for in vivo (mouse exposure) experiments, or cell culture media (see below) for in vitro (lymphocyte exposure) experiments.

Animal Treatment—In Vivo Experiments

Two treatment regimes were used in two different experiments. In the short-term exposure experiments, 6 groups of 10 mice each (5 female, 5 male) were injected intraperitoneally (ip) daily with PBS (diluent control), 10, 25, 50, 100, or 150 mg/kg UDMH. On the fifth day of injections, they were immunized ip with 0.2 ml of a 10% suspension of sheep erythrocytes (SRBC). Five days later, the mice were killed. For the long-term exposure experiment, 5 groups of mice (10 mice per group, 5 male, 5 female) were injected ip 3 days per week for 14 weeks with PBS (diluent control), 25, 50, 100, or 150 mg/kg UDMH. Four days before sacrifice, the mice were immunized intravenously (iv) with 0.2 ml of a 10% suspension of SRBC.

Abbreviations. UDMH: 1,1-dimethylhydrazine; PBS: phosphate buffered saline; SRBC: sheep red blood cells; ip: intraperitoneally; iv: intravenously; HBSS: Hank's balanced salt solution; CCM: cell culture medium; LBT: lymphocyte blast transformation; Con A: concanavalin A; LPS: lipopolysaccharide; cpm: counts per minute; C: complement; SEM: standard error of the mean; NS: not significant; ND: not done; IgG: immunoglobulin G; IgM: immunoglobulin M; EDTA: ethylenediaminetetraacetic acid; ^{3}H-TdR: tritiated thymidine; SLE: systemic lupus erythematosus.

Spleen Cells

Mice were killed by cervical dislocation, and the spleens aseptically removed and placed in L-15 media (Grand Island Biological Co., Grand Island, NY) supplemented with 1% ethylenediaminetetraacetic acid (EDTA) and 1% antibiotic solution. The spleens were separately teased, strained through #60 wire mesh, and aspirated several times through 18, 25, and 27 gauge needles. The red cells were lysed with 0.15 M ammonium chloride lysing solution, and the white cells were then washed and suspended in cell culture medium (CCM) consisting of RPMI 1640 (Grand Island Biological Co., Grand Island, NY), supplemented with 2×10^{-4} M 2-mercaptoethanol, 10% fetal bovine serum (Sterile Systems, Inc., Logan, UT), and 1% antibiotic solution. The splenocytes were counted and diluted to 10^6 lymphocytes/ml. Viability was 97–100%. Percent of lymphocytes ranged from 85–99% (other cells being macrophage or neutrophils).

Lymphocyte Blast Transformation (LBT) Assay

Microtiter plates (CoStar, Cambridge, MA) were filled with 0.1 ml cell suspension (10^5 lymphocytes) per well. Concanavalin A (Con A) (Sigma Chemical Co., St. Louis, MO) and lipopolysaccharide (LPS; derived from *E. coli* 0127:B8; Difco Laboratories, Detroit, MI) were added to murine splenocytes in 10 μl aliquots (0.2 μg/well of Con A and 10 μg/well of LPS). Quadruplicate wells were set up for each mitogen and CCM control. Cultures were incubated at 37°C in a 5% CO_2 humidified incubator for 54 hr, then pulsed with 0.5 μCi/well of tritiated thymidine (^{3}H-TdR; New England Nuclear, Boston, MA) and harvested 18 hr later using a multiple automated sample harvester (Otto Hiller Co., Madison, WI). The glass fiber disks containing the harvested cells were dried and placed in vials with scintillation cocktail. Counts per minute (cpm) were obtained using a liquid scintillation counter (Packard Tricarb Model 3375, Packard Instrument Co., Downers Grove, IL). "Corrected cpm" were determined by (cpm of mitogen-stimulated cultures) – (background cpm [cultures with CCM]).

UDMH Treatment of Spleen Cells—In Vitro LBT Experiments

Spleen cells were harvested as described from 24 normal mice. UDMH was added to LBT cultures containing Con A, LPS, or CCM in concentrations of 0 (control), 5, 10, 25, 50, 75, 100, or 150 μg/ml. UDMH was present throughout the incubation period. The LBT assay was carried out as described above.

Jerne Plaque Assay

The agar-free slide modification described by Cunningham (1965) was used in all assays. Briefly, SRBC were washed and diluted to a 15% suspension in PBS. Spleen cells were harvested as described from SRBC immunized mice (treated with UDMH or PBS), and suspended in RPMI 1640 with 0.5% gelatin (Sigma Chemical Co., St. Louis, MO) at 3×10^6/ml. Guinea pig complement (C) (Grand Island Biological Co., Grand Island, NY) was reconstituted and absorbed with SRBC, if necessary. Rabbit anti-mouse immunoglobulin (Ig)G (Miles Laboratories, Elkhart, IN) was reconstituted with sterile water, absorbed with SRBC, and frozen in 100 μl aliquots. For direct plaque assays (IgM secreting cells), 30 μl of a mixture of 0.3 ml spleen cells, 0.04 ml SRBC suspension, 0.04 ml media, and 0.02 ml C was placed in Cunningham chambers. For indirect plaque assays, 0.04 ml media was replaced with 0.04 ml anti-mouse IgG (1 : 40 dilution of stock solution). Each test mixture was run in quadruplicate. Controls consisted of 1) cells, SRBC, and media; 2) cells, SRBC, and anti-mouse IgG; 3) SRBC

and C; and 4)SRBC, anti-mouse Ig, and C. Slides were sealed with petroleum jelly, incubated at 37°C for 1 hr, then refrigerated until reading. Plaques were counted on a dissecting microscope using indirect light. Data were expressed as numbers of plaques per 10^6 spleen cells.

Con A-Induced Suppressor Cell Assay

Spleen cells were harvested as described. Cells from UDMH-treated mice were incubated with Con A (1 μg/ml) or CCM for 48 hr, then washed, incubated with mitomycin C (50μg/10^7 cells) (Sigma Chemical Co., St. Louis, MO) for 30 min at 37°C and washed three times in Hank's balanced salt solution (HBSS; Grand Island Biological Co., Grand Island, NY) with 0.3 M α-methyl-d-mannoside. The number and percent of live cells was determined, and the cells were suspended in CCM at 3×10^6 live cells/ml. One tenth milliliter of this Con A-induced suppressor cell population or the control cell population (incubated in CCM) was added to 0.1 ml of fresh spleen cells (3×10^6/ml) collected from a normal (untreated) donor mouse as previously described. Con A, LPS, or CCM was then added to the cell cultures (quadruplicate wells for each mitogen and cell combination), and they were incubated and harvested as described for the LBT assay. The percent suppression was determined by

$$100 - \frac{\text{cpm of cultures with suppressor (Con A-induced) cells}}{\text{(cpm of cultures with control (media-incubated) cells}} \times 100$$

For the in vitro UDMH experiments, suppressor cells from normal mice were induced by Con A as described, but in the presence of 25 μg/ml UDMH or CCM. The rest of the procedure was carried out as described. The suppressor cell activity generated in the presence of UDMH was compared to that generated by Con A alone.

Cell Viability

Cell viability was determined by the trypan blue dye exclusion method. Percent viability was determined by

$$100 \times \text{(no. live cells)/(no. total cells)}$$

Statistics

For all in vivo experiments, values from UDMH-treated groups were compared to those of the control (PBS-treated) group, and evaluated for significant differences by Student's *t* test. For the in vitro tests, values from UDMH-treated cells were expressed as percent of the control (untreated cell) values and significant differences were evaluated using the paired *t* test.

RESULTS

In vivo Experiments—UDMH-treated Mice

Jerne plaque formation Direct and indirect plaque responses were determined 5 days after ip immunization in the short-term exposure experiment. The 50 and 100 mg/kg dose groups formed more indirect plaques than the control group (1557 ± 516 and 1762 ± 648, respectively, compared to 1011 ± 433 for the control group), although these increases were not statistically significant (Table 1). No significant or consistent change was seen in the direct plaque response. In the long-term exposure experiment, direct (IgM) plaques were measured 4 days after iv SRBC immunization. Mice in the 10 and 50 mg/kg dose groups showed a significantly

higher response than controls ($p < 0.001$) (Table 1). Responses of the higher dose groups (100, 150 mg/kg) were the same as those of the control group.

Con A-induced suppressor cells Both short- and long-term UDMH exposure caused a decreased induction of suppressor cell activity, as seen in Table 2, although the decreases were not statistically significant. The 50, 100, and 150 mg/kg dose groups were most affected in both experiments, manifest by less suppression of the fresh cell response to Con A and actual enhancement of the response to LPS.

Table 1 Effects of short- and long-term UDMH exposure on Jerne plaque formation

	Dose UDMH (mg/kg)	No. PFC/10⁶ spleen cells	
		Direct (± SEM)	Indirect (± SEM)
Short-term exposure[a]	Control (PBS)	296±91	1011±433
	10	179±66	865±317
	25	214±60	1126±385
	50	179±43	1557±516
	100	262±74	1762±648
Long-term exposure[b]	Control (PBS)	1110±340	
	10	1880±430[c]	
	50	1860±430[c]	
	100	1380±320	
	150	1190±120	

[a]Mice treated daily for 9 days and immunized with SRBC 5 days before sacrifice.

[b]Mice treated 3 times weekly for 14 weeks and immunized with SRBC 4 days before sacrifice.

[c]$p < 0.001$.

Table 2 Effects of short- and long-term UDMH exposure of Mice on suppressor cell induction by Con A

	Dose UDMH (mg/kg)	Percent suppression of fresh cell response to:	
		Con A (± SEM)	LPS (± SEM)
Short-term exposure[a]	Control (PBS)	49.2±12.9	7.4±11.8
	10	59.6±7.6	10.7±5.4
	25	37.7±20.0	4.7±11.9
	50	36.5±18.4	−17.9±12.9
	100	30.5±9.7	−9.9±10.4
	150	30.7±24.9	−11.9±10.4
Long-term exposure[b]	Control (PBS)	48.4±11.2	20.9±8.2
	10	42.1±14.4	11.4±11.9
	50	24.9±15.7	−35.5±43.1
	100	47.3±13.3	7.5±10.2
	150[c]	55.7	6.4

[a]Mice treated daily for 9 days and immunized with SRBC 5 days before sacrifice.

[b]Mice treated 3 times weekly for 14 weeks and immunized with SRBC 4 days before sacrifice.

[c]Only one animal tested.

LBT assay None of the UDMH-treated groups showed either suppressed or elevated LBT response to mitogens compared to the controls. All groups showed good responses to both Con A and LPS (data not shown).

Toxicity None of the mice in the short-term study died or showed any signs of toxicity. In the long-term study, 7 out of 10 mice in the highest dose group (150 mg/ml) died by the end of the study; the other three appeared healthy. All mice surviving at the end of the experiment were necropsied and selected tissues were examined histologically; no significant gross or histologic abnormalities were present.

Effect of In Vitro Exposure of Cells to UDMH

LBT assay The LBT response of normal cells to Con A was suppressed by UDMH at concentrations of 25 to 150 μg/ml (Fig. 1). The response to LPS was significantly enhanced at 10 and 25 μg/ml, and depressed at 100 to 150 μg/ml. The suppression of the LPS response was not due to decreased cell viability as a result of UDMH exposure (Table 3), however, the viability of the cultures stimulated with Con A was somewhat decreased at 50 to 150 μg/ml UDMH.

Effect of UDMH on in vitro Con A-induced suppressor cell activity The UDMH inhibited Con A-induced suppressor cell activity in the LBT assay. The suppression of the fresh cell LBT response to Con A in three separate experiments was decreased by an average of 17.2% ($p <$

Figure 1 Effects of UDMH on the LBT response of normal splenocytes to Con A and LPS (vertical bars represent standard error of the mean). (●) Con A; (■) LPS. (a) $p < 0.001$; (b) $p < 0.005$; (c) $p < 0.01$ (paired t test).

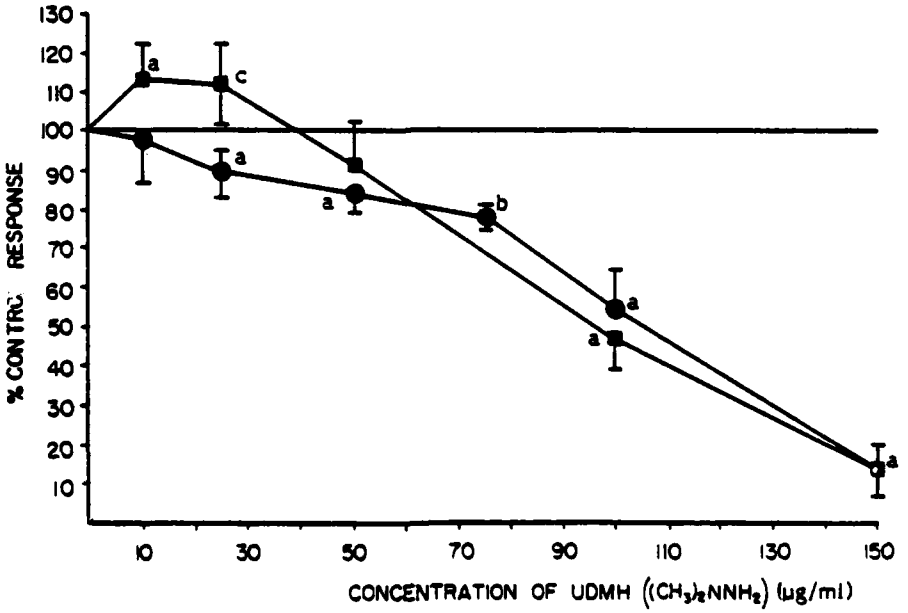

Table 3 Viability of splenocytes incubated with UDMH and Con A or LPS

Concentration UDMH (μg/ml)	Con A % viability (± SEM)	Con A p[a]	LPS % viability (± SEM)	LPS p
0 (control)	86.5±2.2	—	91.5±1.5	—
10	80.5±4.6	NS[b]	95.5±0.5	>0.025
50	67.0±6.5	<0.025	92.0±2.5	NS
100	68.3±8.0	<0.05	89.0±1.9	NS
150	68.5±3.9	<0.005	78.0±3.5	<0.01

[a]p value determined by Student's t test.
[b]NS = not significant.

0.05), and to LPS by 29% (NS), when UDMH (25μg/ml) was present during suppressor cell induction (Table 4).

DISCUSSION

Both the in vivo and in vitro experiments suggest that UDMH inhibits suppressor cell function. Direct and indirect Jerne plaque formation were increased in UDMH-treated mice, and Con A-induced suppressor cell activity was diminished in treated mice. The in vitro studies indicated that UDMH caused an enhanced response to the mitogen LPS, and also abrogated suppressor cell induction in normal spleen cells. In the treated mice as well as in culture, it took relatively low doses of concentrations of UDMH to exert these effects, while higher amounts were similar to controls. This indicates that the suppressor cell population is preferentially inhibited at low doses or that the suppressor-helper ratio is altered in favor of helper cell activity (increased helper activity or decreased suppressor activity). Higher in vivo doses apparently do not affect this ratio, nor are they immunosuppressive; however, in vitro exposure of lymphocytes to higher concentrations of UDMH causes inhibition of the LBT response and toxicity at even higher concentrations.

Inhibition of suppressor cell function would correlate with the observed SLE-like syndrome induced by hydralazine and hydrazine sulfate. It is well-established that natural cases of SLE (Bresnihan and Jasin, 1977) as well as other autoimmune diseases such as Graves' disease (Aoki et al., 1979) or multiple sclerosis (Reinherz et al., 1980) are associated with a decrease in suppressor cell function. More recently, drug-induced autoimmune disorders have been

Table 4 Effects of UDMH in cultures of in vitro suppressor cell induction by Con A

	Percent suppression of fresh cell LBT response to: Con A				LPS			
	Exp. 1	Exp. 2	Exp. 3	Mean	Exp. 1	Exp. 2	Exp. 3	Mean
Suppressor cells induced in presence of UDMH	7.2	−5.1	32.2	11.4 ($p < 0.05$)[a]	59.3	19.3	−95.3	−5.6 NS[b]
Suppressor cells induced alone (control)	19.9	21.4	44.7	28.7	67.5	24.2	−21.4	23.4

[a]p value from paired t test.
[b]NS = not significant.

associated with decreased suppressor T-cell function, such as that reported for methyldopa-induced autoimmune hemolytic anemia (Kirtland et al., 1980). In patients taking methyldopa, polyclonal antibody production was increased compared to normal subjects, and suppressor T-cell activity of cells from these patients on normal cell polyclonal antibody production was diminished. Cyclophosphamide at certain dose levels will also abrogate suppressor T-cell function (Schwartz et al., 1978).

Certainly, other mechanisms may explain the enhancement effects of UDMH. Being a small and unstable molecule, it may alter membrane permeability and ion flux, enhancing the mitogenic stimulus. It is known to act as a growth promotor for fibroblasts at low concentrations (Blakeslee, unpublished data) and it caused somewhat higher background counts in our lymphocyte cultures, although a dose effect was not seen.

The immunoenhancement effects of UDMH could also be related to the interaction of the hydrazine moiety with pyrimidine and/or purine bases of DNA. Dubroff and Reid (1980) have demonstrated several "altered nucleoside products" as a result of incubation of thymidine or deoxyclytidine with hydralazine; and Shank (1980) reported that rats treated with hydrazine showed increased methylation of guanine DNA in the liver. These interactions could result in either acquired immunogenicity of host DNA or, if lymphocyte DNA is affected, abrogation of immune tolerance.

The effects of UDMH, at least partially, may also be genetically determined. Hydralazine-induced SLE occurs nearly exclusively in patients with the "slow acetylator" phenotype (Perry et al., 1970), and the reported case of hydrazine sulfate-induced SLE (Durant and Harris, 1980) was also in a person with the slow acetylator phenotype. It would be of interest to determine the relationship, if any, between acetylase activity and lymphocyte function and interactions.

Regardless of the mechanism, it is apparent that subtoxic doses of hydrazine compounds may enhance the immune response and cause autoimmune disease. This is an important observation, since the hydrazine moiety is prevalent in the environment, found in mycotoxins in common edible mushrooms (Toth et al., 1978), herbicides (maleic hydrazine) (Zurkel, 1957/1963), and missile propellants (such as UDMH) (Beck and Thomas, 1970). It is also present in such commonly used drugs as hydralazine, isoniazid, and hydrazine sulfate. Many hydrazine compounds are known to possess overt toxic or carcinogenic properties, but immunotoxic effects may occur earlier and at lower exposure levels.

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THE EFFECTS OF 1,1-DIMETHYLHYDRAZINE ON THE MURINE MIXED LYMPHOCYTE REACTION

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Abstract:

The in vivo and in vitro effects of 1,1-dimethylhydrazine (UDMH) on the murine one-way mixed lymphocyte reaction (MLR) were examined. In vivo studies in which splenocytes from mice injected with UDMH for one week were used as the responder cell population showed a significantly reduced MLR with doses of 10-50 mg/kg UDMH. The in vitro incorporation of UDMH throughout the MLR 7 day culture period resulted in a concentration related suppression of the MLR of both intact responder cell populations and responder cell populations from which the adherent cells had been removed. Two hour pre-treatment with UDMH on various cell sub-populations involved in the MLR produced the following results: 1) pre-treatment of nonadherent responder cells (without adherent cell component) resulted in a slight concentration related suppression of MLR; 2) pre-treatment of adherent cell component of the responder cell population resulted in an enhancement of MLR; 3) pre-treatment of intact responder cell population, nonadherent cells reconstituted with untreated adherent cells, and intact stimulator cells did not alter MLR. There was no cytotoxicity, due to UDMH treatments, observed in any assays. This data indicates that UDMH, in some manner, modifies specific immune cell functions.

The effects of hydrazine and hydrazine derivatives on a variety of organisms ranging from bacteria to laboratory animals have been studied from a biochemical and genetic viewpoint (Balo [1979], Kimball [1977], Ray, Hanson & Lardy [1970], Smith [1965]); however, little work has been done regarding the possible adverse effects these compounds produce on the immune system. Current research by Tarr et al., has demonstrated that in vivo and in vitro exposure of immunocytes to 1,1-dimethylhydrazine (UDMH) altered their immune responses. In vitro incubation of murine splenocytes with UDMH resulted in a concentration related suppression of concanavalin A (ConA) T-cell mitogenesis, but had a biphasic effect on lipopolysaccharide (LPS) B-cell mitogenesis, that is, enhancing the response at low concentrations while inhibiting the response at higher concentrations. UDMH in vivo and in vitro exposure resulted in inhibition of ConA-induced suppressor activity. Also, splenocytes from UDMH treated mice formed more Jerne plaques than splenocytes from untreated control mice. These altered immune responses suggest that UDMH interferes with immunoregulatory function (Tarr, Olsen & Jacobs [1982]).

Clinical evidence also suggests that hydrazine compounds interfere with immune regulation. Hydralazine, a drug used for treatment of hypertension, has been associated with the occurrence of a systemic lupus erythematosus-like (SLE) syndrome in some patients undergoing this drug therapy. This association is significant because SLE is an autoimmune disease associated with abrogation of suppressor cells and/or suppressor cell function (Dubroff & Reid [1981]). Other hydrazine-containing drugs such as isoniazid, procainamide (Reidenberg [1981]), and hydrazine sulfate (Durrant & Harris [1980]) have also been associated with SLE.

We studied the effects of UDMH on different cell functions in the murine mixed lymphocyte reaction (MLR) with special emphasis on regulator cell activity. Briefly, the MLR is known to occur when a certain subpopulation of T-cells recognizes foreign Ia antigens on a genetically different cell population and reacts by mounting an immune response (Klein [1975]). The T-cells responsible for initial Ia recognition and initiation of MLR are contained in the nonadherent cell population (Boehmer [1974]), while regulatory cell (suppressor cell) activity is at least partly associated with the adherent cell population (Folch & Waksman [1974]). By treating stimulator cells, responder cells, and adherent and nonadherent cell populations within the responder population with UDMH, we hoped to determine this chemical's effects on: 1) cell recognition, 2) response to that recognition, and 3) adherent suppressor cell function.

MATERIALS AND METHODS

Animals The mice used in the MLR assays were Balb C (for stimulator cells) and C57 Bl/6 (for responder cells), aged 6 weeks to 6 months, obtained through the Ohio State University Animal Procurement Service, Columbus, Ohio.

Unsymmetrical Dimethylhydrazine (UDMH) UDMH was obtained from Aldrich Chemical Corporation in a liquid form and purified by distillation by Dr. Kenneth Back and associates, Wright Patterson Air Force Base, Dayton, Ohio. The UDMH was diluted to proper concentrations with culture medium (in vitro experiments) or phosphate buffered saline (PBS) (in vivo administration).

Culture Medium Culture medium consisted of RPMI 1640 with 25 mM hepes buffer (Grand Island Biological Co., Grand Island, N.Y. [GIBCO]) supplemented by 2×10^{-5} M 2-mercaptoethanol, 2.5% pooled human sera (type AB-, GIBCO), 1% L-glutamine (GIBCO), and 1% antibiotic solution consisting of 100 ml Hank's Balanced Salt Solution, 2000 units penicillin, 1 g Streptomycin, 33,333 units of mycostatin, and 1% sodium bicarbonate.

Lymphocyte Isolation Mice were sacrificed by cervical dislocation and their spleens aseptically removed. Single cell suspensions were obtained by straining the spleens through a 60 mesh brass screen into L-15 medium(GIBCO) supplemented with .05% ETDA and 1% antibiotic solution. Cells were then aspirated through a series of 18, 25, and 27 gauge needles. A hemolyzing agent of .15 M ammonium chloride was used to lyse residual erythrocytes in the splenocyte preparation. The cell suspension was then washed with L-15 and suspended in culture medium. Lymphocytes were counted with a hemocytometer and viabilities determined by use of trypan blue stain.

One-Way Mixed Lymphocyte Reaction Procedure Splenocytes from C57 Bl/6 mice were used as the responder cell population. These lymphocytes were suspended at 2.5×10^6 cells/ml in culture medium. Modifications of the responding cell population are described below. Allogeneic stimulator cells were obtained by incubating 1×10^7 splenocytes from Balb C mice with 50 µg mitomycin C (Sigma Chemical Company, St. Louis, Mo.) for 30 minutes at 37° C and then washing three times with cold Hank's Balanced Salt Solution (HBSS, GIBCO). The cells were resuspended in culture medium at a concentration of 5×10^6 cells/ml. C57 Bl/6 cells were also treated in this manner to be used as autologous control stimulator cells. Modifications of the stimulator cell population are described below.

To test for MLR, 100 µl (5.0×10^5) allogenic stimulator cells and 100 µl (2.5×10^5) responder cells were plated in quadruplicate into round-bottomed microtiter plates (Linbro, Flow Laboratories, Inc., Hamden, Conn.). The incorporation of UDMH into the MLR assay throughout the 7 day incubation period was accomplished by plating (in quadruplicate) 50 µl of responder cells (2.5×10^5 cells) and 50 µl of stimulator cells (5.0×10^5 cells) and then adding 100 µl of UDMH per well. The final dilutions of UDMH used were 0, 5, 10, 25, and 50 µg UDMH/ml.

Controls were set up in quadruplicate as follows: 1) responder cells and responder cells, 2) responder cells and autologous stimulators (autologous controls), 3) responders and medium, 4) responders and dilutions of UDMH, 5) allogeneic stimulators and medium, 6) allogeneic stimulators and dilutions of UDMH, 7) autologous stimulators and medium, 8) autologous stimulators and dilutions of UDMH, and 9) allogeneic stimulators and autologous stimulators. The test and control cultures were incubated for seven days at 37° C in a 5% CO_2 humidified atmosphere. Eighteen hours prior to termination, the cells were labeled with 0.5 μCi/well ^{3}H-TdR (New England Nuclear, Boston, MA.) The tests and controls were harvested with a multiple automated sample harvester (Otto Hiller Company, Madison, WI) and after 1 hour drying time the glass fiber disks containing the harvested cells were placed in vials with scintillation cocktail and counted with a liquid scintillation counter (Packard Tricarb Model 3375, Packard Instruments Company, Downers Grove, IL) to determine ^{3}H-TdR incorporation by counts per minute (cpm). Results were recorded as corrected cpm which were obtained by subtracting the autologous control cpm from the MLR test cpm.

Cell Viability Viabilities were obtained by trypan blue dye exclusion test.

Modifications of Responding Cell Populations

Removal of adherent cells from responding cell population To assay the effects of UDMH on the MLR in which the adherent cells were removed from the responding cell population, the following procedure was employed. Responder cells at a concentration of $5x10^6$ cells/ml were placed for 2 hours in 60x15 mm plastic petri dishes (Falcon Labware, Oxnard, CA.). The nonadherent cells were then removed by gently washing 3 times with culture medium. The nonadherent cells were counted and brought to a concentration of $5x10^6$ cells/ml. Responder and stimulator (intact spleen cell population) cells were plated with UDMH and the assay completed as described above.

UDMH pre-treatment of nonadherent cells in responding cell population To test the effects of UDMH treatment on nonadherent cells in the responding cell population, responder cells were prepared as follows. The nonadherent cell population was removed as described above and incubated for two hours at 37° C with 0, 5, 10, 25, or 50 µg/ml of UDMH. The cells were then washed with HBSS, brought to a concentration of 2.5×10^6 cells/ml culture medium, plated with stimulators and the assay completed as described above (without the 7 day incorporation of UDMH).

Also, nonadherent cells were treated with UDMH as described above and then reconstituted with non-treated adherent cells. (Reconstitution procedure follows.) These cells were then used as responders, plated with stimulators and the assay completed as described above.

UDMH treatment of adherent cells in responding cell population To determine the effect of UDMH pretreatment on the adherent cell population, responder cells were prepared as follows. The nonadherent cell population was removed as described above. The adherent cell population was treated for two hours with 0, 5, 10, 25, or 50 ug/ml UDMH and then washed with phosphate buffered saline (PBS). The adhered cells were removed from the petri plates by a 30 minute incubation at 37°C with PBS lacking calcium and magnesium ions, scraping the plates with a sterile rubber policeman, and vigorously washing five times with culture medium. The adherent and nonadherent cells were reconstituted, brought to a concentration of 2.5×10^6 cells/ml medium, plated with stimulators, and the assay was completed as described above.

Modification of stimulator cell populations The allogeneic and autologous stimulator cells were mitomycin C treated as described above, then washed and incubated with 0, 10, 25, 50 and 100 µg/ml UDMH for 2 hours at 37°C. They were then washed, suspended to a concentration of 5×10^6 cells/ml, plated with non-treated responder cells, and assayed as described above.

MLR testing with mice exposed in vivo to UDMH C57Bl/6 mice were injected daily intraperitoneally with 10, 25, or 50 mg/kg doses of UDMH or PBS for one week. The mice were then sacrificed and their splenocytes used as responder cells in the MLR assay.

RESULTS

Effects of In Vitro UDMH on the MLR of Intact and Nonadherent Responder Cell Populations The presence of UDMH throughout the culture period in the MLR of intact responder cells and nonadherent responder cells (i.e., in which the adherent cells had been removed) caused a significant concentration-related suppression in both cases as compared to their medium controls. In the first case (intact MLR), the medium control response was 1271 $\pm$ 351 (standard error of the mean) cpm. The addition of 10 µg/ml, 25 µg/ml, and 50 µg/ml UDMH to the MLR resulted in responses of 822 $\pm$ 315 cpm ($p < .01$), 121 $\pm$ 216 cpm ($p < .005$), and 412 $\pm$ 233 cpm ($p < .025$) respectively (Table 1). The MLR responses of the nonadherent responder cells were also slightly suppressed by UDMH compared to the medium control: 2679 $\pm$ 741 cpm (media); 1507 $\pm$ 607 (5 mg/ml, not significant[N.S.]); 2032 $\pm$ 643 cpm (10 µg/ml, N.S.); 1327 $\pm$ 210 cpm (25 µg/ml; $p < .025$); and 1223 $\pm$ 643 cpm (50 µg/ml; $p < .05$).

Removal of the adherent cells from the responder cell population (leaving nonadherent cells only) resulted in enhanced MLR responses compared to those of the intact responder cell population regardless of the presence of UDMH (Table 1). The responses were significantly greater in medium ($p < .05$); 10 µg/ml UDMH ($p < .05$); 25 µg/ml UDMH ($p < .001$); and 50 µg/ml ($p < .05$).

The Effects of Two Hour Pre-Treatment with UDMH on the MLR of Various Cell Subpopulations Differing cell populations within the MLR were treated with 5, 10, 25, and 50 µg/ml UDMH for 2 hours, washed once, and then assayed for their MLR. The results were as follows:

1. Two-hour pre-treatment of the intact responder cell population with UDMH resulted in no significant alteration of response. The cpm obtained for medium controls 5 µg UDMH/ml, 10 µg/ml, 25 µg/ml and 50 µg/ml were 981 ± 379 cpm, 1077 ± 115 cpm, 1038 ± 146 cpm, 813 ± 85 cpm, and 753 ± 112 cpm respectively.

2. Removal of the adherent cell population and 2-hour UDMH treatment of the nonadherent cells in the responder cell population produced a concentration-related suppression of the MLR similar to what was observed in the 7 day UDMH exposure MLR assays. The cpm for the medium control were 1878 ± 187, and for UDMH pretreated, nonadherent cells were 1554 ± 352 (5 µg/ml; N.S.); 1304 ± 165 (10 µg/ml; $p < .025$); 1361 ± 280 (25 µg/ml; N.S.); and 1178 ± 304 (50 µg/ml: $p < .05$) (Table 2).

The removal of the adherent cells also resulted in an increased MLR response of the pretreated nonadherent cells compared to pretreated intact cells as was seen when UDMH was present throughout the culture period. Compared to the cpm of the intact responder cells, the cpm of nonadherent responder cells were significantly increased in media ($p < .05$), 5 µg/ml UDMH ($p < .05$) and 25 µg/ml ($p < .025$) (Table 2).

3. UDMH pretreatment of nonadherent cells followed by reconstitution with normal adherent cells of the responder cell population had no effect on the MLR response. The cpm obtained were 1834 ± 605 (media); 1551 ± 281 (5 µg/ml UDMH); 2209 ± 649 (10 µg/ml UDMH); 1750 ± 320 (25 µg/ml UDMH); and 1571 ± 510 (5 µg/ml UDMH) (Table 2).

4. Two hour UDMH pretreatment of adherent cells in the responder cell population followed by reconstitution with normal (untreated) nonadherent cells resulted in a mild increase in the MLR as compared to medium controls. The cpm of the control cultures (media) were 1628 ± 499, compared to 2287 ± 628 (5 µg/ml; N.S.), 1944 ± 628 (10 µg/ml; N.S.), 2244 ± 583 (25 µg/ml; N.S.), and 2658 ± 657 (50 µg/ml; $p < .05$) (Table 2).

These responses were also significantly greater than the responses of UDMH-treated intact responder cells (5 μg/ml, $p < .05$; 10 μg/ml, $p < .05$; 25 μg/ml, $p < .005$; and 50 μg/ml, $p < .005$) (Table 2).

5. Two hour UDMH pretreatment of the intact stimulating cell population caused no significant alteration in the MLR of normal (untreated) intact responder cells (Table 3).

There was no effect on control cultures due to the presence of UDMH in all of the in vitro assays.

In Vivo Effects of UDMH on the MLR Preliminary experiments indicated that daily injections of UDMH for one week caused significant dose-related suppression on the MLR of treated mice. Compared to PBS-treated controls (4340 ± 929 cpm), the cpm were lower at doses of 10 mg/kg/day (2733 ± 1344; $p < .01$); 25 mg/kg/day (2115 ± 503; $p < .025$); and 50 mg/kg/day (505 ± 42; $p < .005$) (Table 4).

Cell Viabilities The UDMH had no significant cytotoxic effect, as evaluated by trypan blue dye exclusion, in any of the in vitro or in vivo assays.

DISCUSSION

The effects of UDMH on the MLR, an in vitro correlate of cell-mediated immunity, were determined. Our data indicate that in vitro and in vivo exposure of immunocytes to UDMH results in a depressed MLR. Splenocytes from mice injected daily with UDMH for one week showed a significantly reduced MLR with daily doses of 10-50 mg/kg UDMH. Similarly, the presence of UDMH throughout the 7 day culture period resulted in a concentration related suppression of the MLR of both intact responder cell populations and responder cell populations in which the adherent cells had been removed.

Pre-incubation of UDMH with various cell sub-populations within the MLR yielded the following results: 1) UDMH pre-treatment of non-adherent responder cells (without return of the adherent cell component) resulted in a mild concentration-related suppression of the MLR; 2) Pre-treatment of the adherent cell component of the responder cell population resulted in a mild concentration-dependent enhancement of the MLR; 3) UDMH pre-treatment of intact responder cells, non-adherent responder cells reconstituted with untreated adherent cells, and intact stimulator cells did not alter the MLR. These data indicate first of all that the MLR response of the nonadherent cell component of the responder cell population is reduced as a result of exposure to UDMH. One possible explanation for this suppression is interference with Ia (I-region associated) antigen receptor interaction. The MLR occurs because of recognition and response of a non-adherent T-cell population to Ia antigens present on stimulator T and B-cells (Klein [1975]). The UDMH may cause an alteration in Ia antigen receptor sites on the T-cell surface membrane, thereby rendering the T cell incapable of recognition and response. The stimulating capacity of the Ia antigen, however, apparently is not altered because UDMH did not affect the MLR when stimulator cells were pre-treated with UDMH.

Another possible explanation for this suppression is metabolic interference caused by UDMH. In some cell types, carbohydrate metabolism is abrogated due to the presence of UDMH (Ray et al.[1970]) This metabolic defect may in turn affect the immune function of the nonadherent cell.

The responder adherent cell population was also affected by UDMH. Monocytes, the major component of the adherent cell population, are necessary for the MLR to occur. It is thought that these cells function as "communicator" cells between responder and stimulator lymphocytes. Monocytes in the stimulator cell population may also function in this capacity, even though they have been inactivated by mitomycin C. (Mann & Abelson[1980]).

A suppressor or regulator function has also been attributed to monocytes or some other cellular component of the adherent cell population (Rice, Laughter & Twomey [1979]). Our data indicated that the MLR of the nonadherent responder cells alone was significantly higher than the MLR of intact (adherent and nonadherent) responder cells, regardless of the presence of UDMH. These studies also showed that when adherent cells were pre-treated with UDMH and then added back to normal nonadherent cells, the MLR of the reconstituted responder cells was slightly enhanced. Two explanations for this effect exit. First, there is a possibility that UDMH may specifically stimulate monocyte "communicating" cells or another responding cell in the adherent cell population and thereby enhance the MLR. Along these lines, studies in our laboratory have indicated that UDMH enhances phagocytic and microbicidal properties of peritoneal macrophages during a 2-hour incubation period, indicating a non-specific activation of macrophages (unpublished data). A second and more conceivable theory is that UDMH specifically inhibits the suppressor activity of the adherent cell population, resulting in an increased MLR when the adherent cells alone are exposed to UDMH. Other experiments in our lab which support a UDMH-induced abrogation of suppressor activity include: 1) low concentrations of UDMH caused an enhanced lymphoblast transformation response to lipopolysaccharide; 2) mice treated with UDMH responded to sheep erythrocyte immunization with increased numbers of plaque-forming cells as compared to control mice; and 3) in vivo and in vitro UDMH treatment inhibited concanavalin-A induced suppressor activity (Tarr et al. [1982]).

A possible mechanism for the loss of suppressor activity in the MLR could again be UDMH-induced interference with membrane Ia antigen-receptor interaction. Cell-mediated suppressor activity is thought to depend on this interaction, (Zan-Bar, Murphy & Strober [1978]) and UDMH may somehow alter the Ia antigen on the suppressor cells so that they cannot effectively interact with and suppress the responding T-cells.

The likelihood that UDMH interacts with the cell membrane is supported by studies using hydrazine in other in vitro cell culture systems, in which both the morphology (toad kidney cells) and membrane function (myocardial cells) were altered when the cells were incubated with hydrazine. Hydrazine also caused cell fusion, resulting in multinucleated giant cells. (Siemans, Kitzes & Berns [1981]). UDMH is a similar small, reactive compound which most likely behaves similarly to hydrazine.

In conclusion, it is clear that UDMH alters the MLR both in vivo and in vitro, and the nature of the alterations in vitro depends on which subpopulation of cells is exposed to UDMH. Studies are now ongoing which will hopefully delineate the site and mechanism of action responsible for these effects.

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Table I. Effects of the presence of UDMH on control MLR and MLR of nonadherent cell population.*

Concentration of UDMH (ug/ml)	Control MLR	p_1 value†	MLR of nonadherent cells	p_1 value	p_2 value‡
0 (media control)	1271 ± 351	- - - -	2679 ± 741	- - - -	<.05
5	1507 ± 607	n.s.	2185 ± 695	n.s.	n.s.
10	822 ± 315	.01	2032 ± 643	n.s.	<.05
25	121 ± 216	.005	1327 ± 210	<.025	<.001
50	412 ± 233	.025	1223 ± 643	<.050	<.05

* Response is recorded as corrected counts per minute(cpm) ± standard error (see text). n = 20-32

† p_1 value - determined by student's t test; values compared to media control within each assay.

‡ p_2 value - determined by student's t test; values compared to control MLR.

Table 2. Effects of 2-hour pre-incubation with UDMH on cell sub-populations in the MLR*

Concentration of UDMH (ug/ml)	Treated responder cells (intact)		Treated nonadherent responder cells (not reconstituted)			Treated nonadherent responder cells (reconstituted)			Treated adherent responder cells (reconstituted)		
		P_1 value†		P_1 value	P_2 value‡		P_1 value	P_2 value		P_1 value	P_2 value
0 (control)	981±379	- -	1878±187	- -	<.05	1834±605	- -	n.s.	1628±499	- -	n.s.
5	1077±115	n.s.	1554±352	n.s.	<.05	1551±281	n.s.	n.s.	2287±628	n.s.	<.05
10	1038±146	n.s.	1304±165	<.025	n.s.	2209±649	n.s.	<.05	1944±628	n.s.	<.05
25	813±85	n.s.	1361±280	<.10	<.025	1750±320	n.s.	<.05	2244±583	n.s.	<.005
50	753±112	n.s.	1178±304	<.05	n.s.	1571±510	n.s.	n.s.	2658±657	<.05	<.005

* Responses recorded as corrected counts per minute ± standard error (see text). n = 20-32

† P_1 value - determined by student's t test; values compared to media control within each assay.

‡ P_2 value - determined by student's t test; values compared to treated responder assay for each UDMH concentration.

Table 3. Effects of 2-hour preincubation of stimulator cells with UDMH*

Concentration of UDMH(ug/ml)	MLR	p value†
0 (media control)	1590 ± 389	- - - -
10	994 ± 204	n.s
25	1631 ± 326	n.s.
50	1416 ± 312	n.s.
100	939 ± 107	n.s.

*Responses recorded as corrected counts per minute ± standard error. (see text) n = 20-32

† p value determined by student's t-test; values compared to media control.

Table 4. Effects of UDMH on MLR when administered in vivo.

Dose (mg UDMH/kg mouse)	MLR*	p value†
No treatment	4577 ± 791	--
PBS	4340 ± 929	n.s.
10	2733 ± 1344	.01
25	2115 ± 503	.025
50	505 ± 42	.005

* Responses recorded as corrected counts per minute ± standard (see text). n = 20-32

† p value determined by student's t-test.